

Cisplatin-loaded Polymer/Magnetite Composite Nanoparticles as Multifunctional Therapeutic Nanomedicine*

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Abstract Multifunctional nanocarriers with multilayer core-shell architecture were prepared by coating superparamagnetic Fe_3O_4 nanoparticles with diblock copolymer folate-poly(ethylene glycol)-*b*-poly(glycerol monomethacrylate) (FA-PEG-*b*-PGMA), and triblock copolymer methoxy poly(ethylene glycol)-*b*-poly(2-(dimethylamino) ethyl methacrylate)-*b*-poly(glycerol monomethacrylate) (MPEG-*b*-PDMA-*b*-PGMA). The PGMA segment was attached to the surfaces of Fe_3O_4 nanoparticles, and the outer PEG shell imparted biocompatibility. In addition, folate was conjugated onto the surfaces of the nanocarriers. Cisplatin was then loaded into the nanocarrier by coordination between the Pt atom in cisplatin and the amine groups in the inner shell of the multilayer architecture. The loaded cisplatin showed pH-responsive release: slower release at pH 7.4 (*i.e.* mimicking the blood environment) and faster release at more acidic pH (*i.e.* mimicking endosome/lysosome conditions). All of the cisplatin-loaded nanoparticles showed concentration-dependent cytotoxicity in HeLa cells. However, the folate-conjugated cisplatin-loaded carriers exhibited higher cytotoxicity in HeLa cells than non-folate conjugated cisplatin-loaded carriers.

Keywords: Drug delivery; Folate; Targeting; Cisplatin; Magnetic.

INTRODUCTION

Nanomedicine has gained momentum over the last few decades due to its potential to improve therapeutic efficacy and imaging capabilities. The core of nanomedicine is the development of nanoparticles (*e.g.*, liposomes, dendrimers, polymeric micelles, magnetic nanoparticles, quantum dots and carbon nanotubes) that function as carriers for therapeutic and diagnostic agents^[1–6]. In both therapeutic and diagnostic applications, one of the most essential requirements for a nanocarrier is efficient drug or imaging agent delivery to the target site. Nanoparticles have a tendency to accumulate in tumors due to the enhanced permeability and retention (EPR) effect that results from leaky tumor neovasculature and the absence of a functioning lymphatic drainage system at the tumor site, referred to as the so-called “passive targeting”. However, nanoparticles tend to adsorb plasma components such as opsonins, leading to rapid plasma clearance of the nanocarriers. Modification of nanoparticle surfaces with neutral and hydrophilic polymers (*e.g.*, poly(ethylene glycol), PEG) can prevent

* This work was financially supported by the National Natural Science Foundation of China (Nos. 51373080, 81001417 and 20974052), PCSIRT (No. IRT1257), the National Key Technologies R & D Program for New Drugs of China (No. 2009ZX09301-002), and the Natural Science Foundation of Tianjin Municipality (No. 09JCZDJC22900).

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Received December 5, 2013; Revised February 24, 2014; Accepted February 27, 2014

doi: 10.1007/s10118-014-1510-1

opsonization, thus prolonging circulation. Recently, multifunctional nano-systems, referred to as theranostics, which combine targeting, imaging and drug delivery capabilities, have shown tremendous promise in cancer therapy^[7, 8]. Nanoparticles are useful platforms in both imaging and therapeutic applications, and have been developed for their potential ability to simultaneously image and treat disease at the cellular level. Because of these multifunctional features, these systems require a suitable structure to incorporate drugs and imaging agents with appropriate surface functionality to enable tumor targeting. Many imaging contrast agents have been incorporated into multifunctional nanocarriers for various imaging modalities, including fluorescence optical imaging, magnetic resonance imaging (MRI), positron-emission tomography (PET) and computed tomography (CT). MRI is one of the most powerful non-invasive imaging techniques in both clinical and basic research fields because of its ability to provide high spatial and temporal resolution. Among various MRI contrast agents, superparamagnetic iron oxide nanoparticles including magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$) superparamagnetic nanocrystals have been intensively studied as promising MRI probes due to their favorable magnetic properties, low toxicity and high chemical stability^[9, 10]. In addition to their role as MRI contrast agents, superparamagnetic iron oxide nanocrystal-containing nanocarriers can also be used as heating mediators for cancer hyperthermia, because magnetic nanoparticles convert energy absorbed from an alternating magnetic field into heat^[11, 12].

Platinum-based anticancer agents such as cisplatin (*cis*-diaminedichloroplatinum) are a mainstay of clinical drugs for the treatment of various solid tumors. They are frequently administered intravenously for the treatment of many malignancies, such as testicular, bladder, ovarian, small cell and non-small cell lung cancer^[13]. However, a major drawback of platinum drugs for clinical applications is the presence of many severe side effects due to drug toxicity in normal tissues, such as acute nephrotoxicity and chronic neurotoxicity. Furthermore, the development of drug resistance by tumors also limits the clinical application. To overcome these drawbacks, platinum drugs can be physically encapsulated in a polymer matrix, but even more attractive for the delivery of platinum drugs is the binding of the drug to the polymer matrix with coordination groups, mostly carboxyl groups, *via* metal-ligand coordination^[14–20]. Platinum drugs are also loaded into multifunctional nanocarriers composed of carboxyl group-containing polymer-coated magnetite nanoparticles^[21–24].

Platinum drugs usually contain ligands with nitrogen donor atoms^[25]. There are a few reports in which amine group-containing dendrimers^[26] and polymer micelles^[27] are used as carriers to load platinum drugs by coordination of the amine groups to the Pt atom. In this paper, we fabricated a multifunctional nanocarrier, which composed of a magnetite nanoparticle core, an amine containing polymer inner shell, a biocompatible PEG corona and folate groups decorated on the surface. Cisplatin was loaded into the inner shell of the nanocarrier by coordination of the amine groups to the Pt atoms. The drug delivery system showed faster release of the loaded cisplatin at more acidic pHs than at pH 7.4 due to the protonation of the amine groups and active targeting property.

EXPERIMENTAL

Materials

Methoxy poly(ethylene glycol) (MeO-PEG-OH, 2000 Da, Aldrich) and poly(ethylene glycol) (HO-PEG-OH, 3000 Da, Fluke) were dehydrated by azeotropic distillation of water in toluene, and vacuum-dried for 24 h at 80 °C. 2-(Dimethylamino) ethyl methacrylate (DMA, Aladdin) was distilled over CaH_2 under reduced pressure before use. 1,1,4,7,7-Pentamethyldiethylenetriamine (PMDETA) was purchased from Acros and used directly. 2-Bromoisobutryl bromide (BIBB) was purchased from Aldrich. Folate (FA) and *o*-phenylenediamine were purchased from Tianjin Guangfu Fine Chemical Research Institute. Solketal methacrylate (SMA) was prepared by reaction of solketal (2,2-dimethyl-1,3-dioxane-4-methanol) with methacryloyl chloride, as described previously^[28]. Cisplatin (CDDP) was purchased from Aladdin. The human cervical carcinoma HeLa cell line was obtained from the Cell Resource Center of Peking Union Medical College. RPMI 1640, RPMI 1640 without folic acid and fetal bovine serum (FBS) were purchased from GIBCO. The CellTiter 96® AQueous One Solution

Cell Proliferation Assay kit [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (PES)] was purchased from Promega.

Block Copolymer Synthesis

Diblock copolymer folate-poly(ethylene glycol)-*b*-poly(glycerol monomethacrylate) (FA-PEG-*b*-PGMA), and triblock copolymers methoxy poly(ethylene glycol)-*b*-poly[2-(dimethylamino) ethyl methacrylate]-*b*-poly(glycerol monomethacrylate) (MPEG-*b*-PDMA-*b*-PGMA) were synthesized by atom transfer radical polymerization (ATRP) as described previously^[28, 29]. The structures of all the synthesized copolymers were confirmed by ¹H-NMR.

Preparation of Polymer-coated Fe₃O₄ Nanoparticles

MPEG-*b*-PDMA-*b*-PGMA-coated Fe₃O₄ nanoparticles (denoted POLYMER-Fe₃O₄) and FA-PEG-*b*-PGMA-/MPEG-*b*-PDMA-*b*-PGMA-coated Fe₃O₄ nanoparticles (denoted FA-POLYMER-Fe₃O₄) were prepared using a ligand-exchange procedure in which an aqueous solution of PEG-*b*-PDMA-*b*-PGMA or FA-PEG-*b*-PGMA-/MPEG-*b*-PDMA-*b*-PGMA (1/9, weight ratio) was mixed with ClO₄⁻-stabilized Fe₃O₄ nanoparticles dispersed in water, followed by dialysis as described previously^[29]. The ratio of copolymer to ClO₄⁻-stabilized Fe₃O₄ nanoparticles was 1/5 (weight ratio).

Drug Loading and In Vitro Release

Cisplatin was loaded into the polymer-coated Fe₃O₄ nanoparticles as follows: an aqueous solution of cisplatin (4 mL, 1 mg/mL) was added dropwise into the polymer-coated Fe₃O₄ nanoparticle dispersion in phosphate buffer (10 mmol/L, pH 7.4). After stirring for 24 h in the dark, the dispersion was dialyzed (*M_w* cut-off 8000–14000 Da) against water for 15 h, changing the external water every 3 h. The cisplatin-loading capacity was estimated by subtraction of the amount of cisplatin in the collected external solutions from the total amount of cisplatin added. The cisplatin concentration was measured by the *o*-phenylenediamine method^[30]: a sample solution was mixed with an *o*-phenylenediamine solution in dimethyl formamide (1.5 mg/mL) at a volume ratio at 1.5/1. The mixture was incubated at 100 °C for 15 min, and the absorbance of the solution at 703 nm was measured by UV-Vis spectrum. The cisplatin-loaded POLYMER-Fe₃O₄ and FA-POLYMER-Fe₃O₄ were denoted CIS-POLYMER-Fe₃O₄ and FA-CIS-POLYMER-Fe₃O₄, respectively.

In vitro release was performed as follows: the cisplatin-loaded nanoparticle dispersion was dialyzed (*M_w* cut-off 8000–14000 Da) against phosphate buffered saline (PBS, 10 mmol/L phosphate, 150 mmol/L NaCl) with different pH values (pH 7.4, 6.5 and 5.7). After 1, 3, 6, 10, 13, 16, 20 and 24 h of dialysis, 5 mL of the external solution was taken for the determination of the concentration of cisplatin, and the same volume of fresh buffer was added. The concentration of the Pt was measured by the method mentioned above.

Cytotoxicity Assay

The human cervical carcinoma HeLa cell lines were grown and maintained in folate-free RPMI 1640 medium or RPMI 1640 medium with the addition of folate (0.35 mg/mL) in a humidified atmosphere with 5% CO₂ at 37 °C. Both types of media used contained FBS (10%), penicillin (100 U/mL) and streptomycin (125 U/mL).

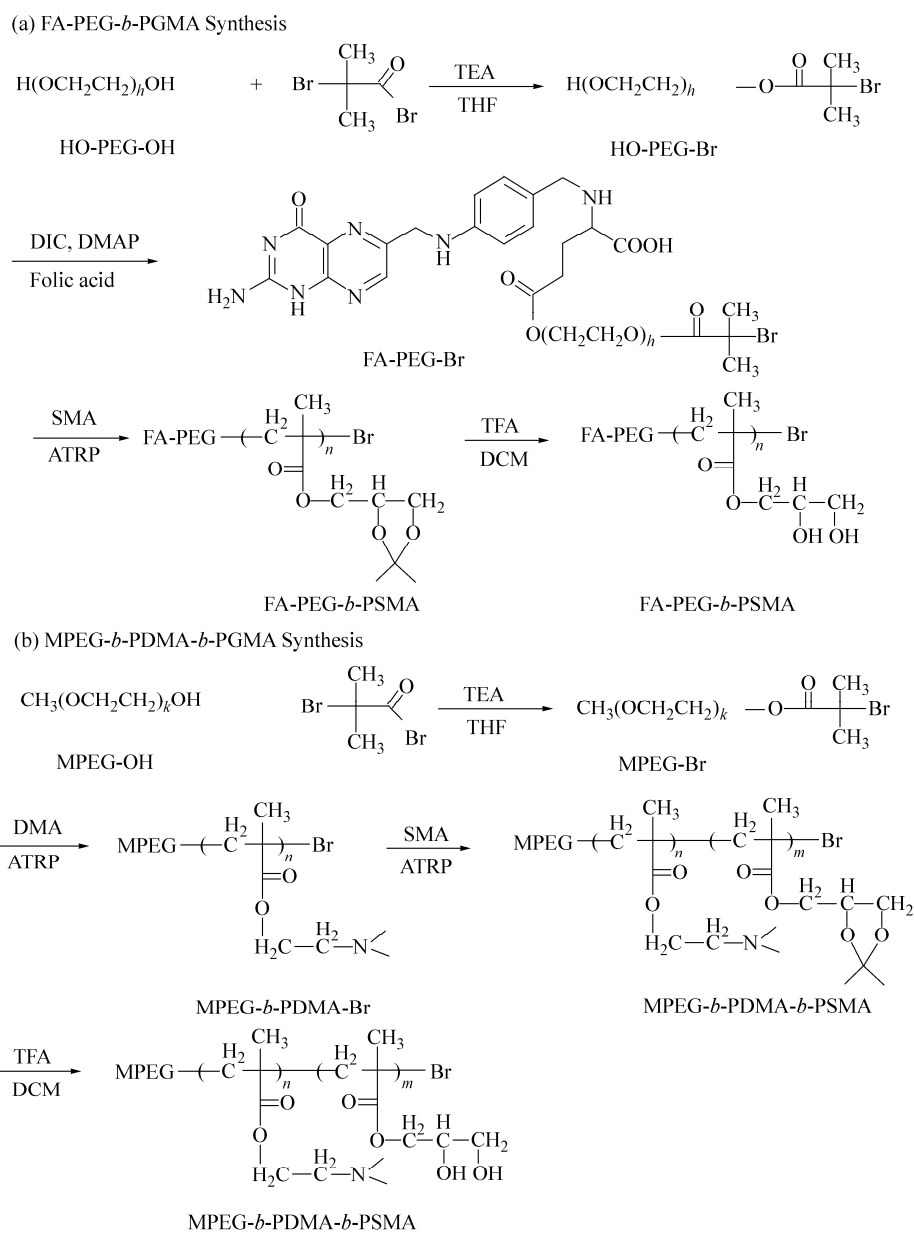
HeLa cells cultured in folate-free RPMI 1640 medium were seeded in a 96-well plate at a density of 1×10^4 cells/well. After 24 h incubation, the medium in the wells was replaced with 200 µL of cisplatin-loaded nanoparticles or free cisplatin (1, 4, 8, 12 µg cisplatin/mL) in PBS. After 1 h incubation, the medium was removed, the cells were washed with fresh PBS, and 200 µL of fresh RPMI 1640 medium was added. The cells were incubated for another 20 h in the humidified atmosphere with 5% CO₂ at 37 °C. To estimate cell survival, media was removed and the cells were washed three times with PBS. 20 µL of the MTS solution and 70 µL of PBS were added to each well of the 96-well plate. After incubation for 3 h in a humidified atmosphere with 5% CO₂ at 37 °C, the absorbance at 490 nm of each well was read using a SpectraMax M5 microplate reader. The spectrophotometer was calibrated to zero absorbance using PBS alone. The relative cell viability compared to control wells containing PBS without nanoparticles was calculated by $[Abs]/[Abs]_0$, where $[Abs]$ and $[Abs]_0$ are the average absorbance of the test and the control samples, respectively.

The cytotoxicity assay in the culture medium containing folate was performed as shown above except that RPMI 1640 medium with the addition of folate (0.35 mg/mL) was used.

RESULTS AND DISCUSSION

Preparation of Polymer-coated Magnetite Composite Nanoparticles

Block copolymers FA-PEG-*b*-PGMA and MPEG-*b*-PDMA-*b*-PGMA were synthesized by ATRP (Scheme 1), and the ^1H -NMR spectra of the precursors and targeted polymers are shown in Fig. 1. The degree of polymerization of the segments of the block copolymers was derived by ^1H -NMR using the MPEG or PEG segment as the reference (Table 1). Narrow molecular weight distribution of the copolymers was confirmed by gel permeation chromatography (GPC) (a combination of columns Styragel HT-2, HT-3 and HT-4, Waters) (Table 1).



Scheme 1 The synthesis process of block polymers: (a) FA-PEG-*b*-PGMA and (b) MPEG-*b*-PDMA-*b*-PGMA

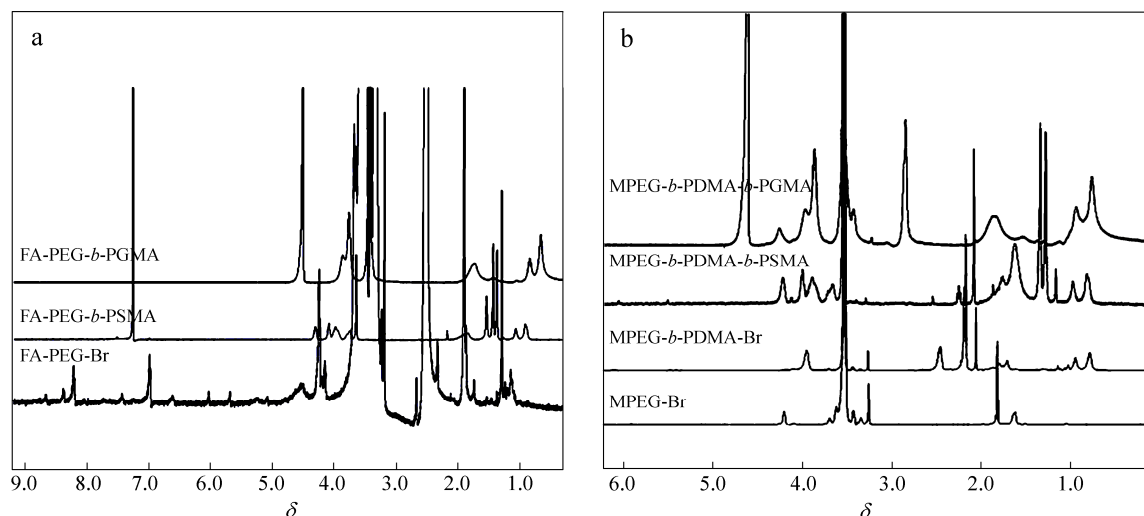


Fig. 1 ^1H -NMR spectra of (a) FA-PEG-*b*-PGMA and (b) MPEG-*b*-PDMA-*b*-PGMA and their precursors

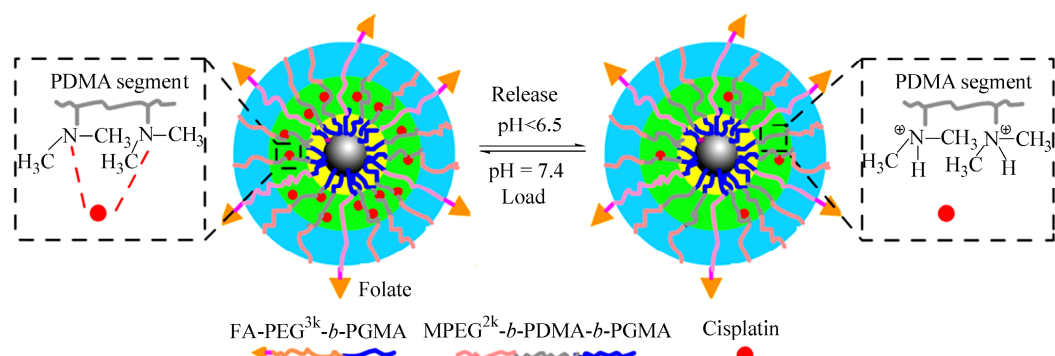
Table 1. Characterization of the block copolymers

Copolymer	DR _{PEG} ^a	DR _{MAA} ^a	DR _{DMA} ^a	DR _{GMA} ^a	M_n (kD) ^b	M_w/M_n ^b
MPEG- <i>b</i> -PDMA- <i>b</i> -PGMA	45	—	17	42	26.7	1.25
FA-PEG- <i>b</i> -PGMA	68	—	—	42	34.1	1.34

^a DR, degree of polymerization. DR_{PEG} was calculated from the average molecular weight of MPEG (2000 Da) or PEG (3000 Da). DR_{PEG}, DR_{MAA}, DR_{DMA} and DR_{GMA} were derived from ^1H -NMR using MPEG or PEG segments as the reference;

^b M_n and M_w represent the number average molecular weight and weight average molecular weight, respectively, of the precursor polymers, MPEG-*b*-P α BMA-*b*-PSMA, MPEG-*b*-PDMA-*b*-PSMA and FA-PEG-*b*-PSMA (see Scheme 1), as determined by GPC.

Polymer-Fe₃O₄ and FA-polymer-Fe₃O₄ were prepared by an indirect method according to our previous study^[29], *i.e.*, by mixing an aqueous solution of MPEG-*b*-PDMA-*b*-PGMA or FA-PEG-*b*-PGMA-/MPEG-*b*-PDMA-*b*-PGMA with ClO₄[−]-stabilized Fe₃O₄ nanoparticles dispersed in water, followed by dialysis against water. These polymer-coated Fe₃O₄ nanoparticles can be stably dispersed in water and PBS. In our previous paper^[29], we demonstrated that PGMA homopolymer and the PGMA segment of PGMA-containing block copolymers such as, PDMA-*b*-PGMA and MPEG-*b*-PGMA can attach tightly to the surfaces of magnetite nanoparticles by coordination of the diol groups to the Fe atoms, while the MPEG or PDMA segment formed the shell of the nanoparticles. Accordingly, the multilayer structure of FA-POLYMER-Fe₃O₄ was deduced and illustrated in Scheme 2. Figures 2(a) and 2(b) show the TEM images of the polymer-coated Fe₃O₄ nanoparticles. The TEM pictures indicated that the particles were roughly spherical or ellipsoidal in shape with some irregularities and a narrow size distribution. From these images, we estimated that the average diameter of the core Fe₃O₄ nanoparticles was ~10 nm. These images did not clearly depict the outer copolymers. The size and size distribution of the nanoparticles were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instruments Ltd), and the results are shown in Figs. 2(c) and 2(d) and Table 2. Table 2 shows that the particle size changed little when cisplatin was loaded into the nanoparticles. All the copolymer-coated Fe₃O₄ nanoparticles showed superparamagnetic behavior without magnetic hysteresis, according to SQUID magnetization at 300 K. The SQUID magnetization pictures of the nanoparticles are shown in Fig. 3. Figure 3 shows that, when the cisplatin was loaded into the nanoparticles, the saturation magnetization intensity decreased a little.



Scheme 2 Schematic representation of the structure of FA-CIS-POLYMER-Fe₃O₄ nanoparticles and cisplatin loading and release

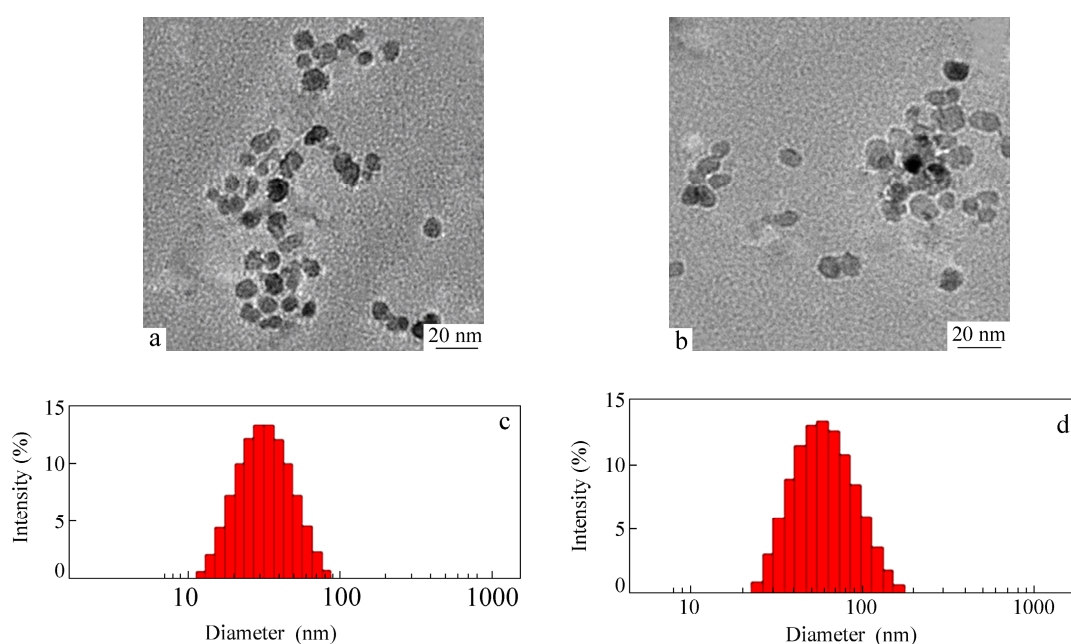


Fig. 2 TEM images of (a) POLYMER-Fe₃O₄ nanoparticles and (b) FA- POLYMER-Fe₃O₄ nanoparticles; DLS graphics of (c) POLYMER-Fe₃O₄ nanoparticles and (d) FA-POLYMER-Fe₃O₄ nanoparticles

Table 2. Size and size distribution of the polymer-coated Fe₃O₄ nanoparticles

Nanoparticles	Size and size distribution		Cisplatin loading capacity± SD (%) ^a
	Size (nm) ± SD ^a	PDI ± SD ^a	
POLYMER-Fe ₃ O ₄	62.85 ± 1.82	0.163 ± 0.002	—
CIS-POLYMER-Fe ₃ O ₄	65.08 ± 1.80	0.174 ± 0.010	5.78 ± 0.62 %
FA-POLYMER-Fe ₃ O ₄	53.09 ± 1.06	0.162 ± 0.005	—
FA-CIS-POLYMER-Fe ₃ O ₄	55.91 ± 2.12	0.170 ± 0.004	5.75 ± 0.60 %

^a Results are means ± SD (*N* = 3).

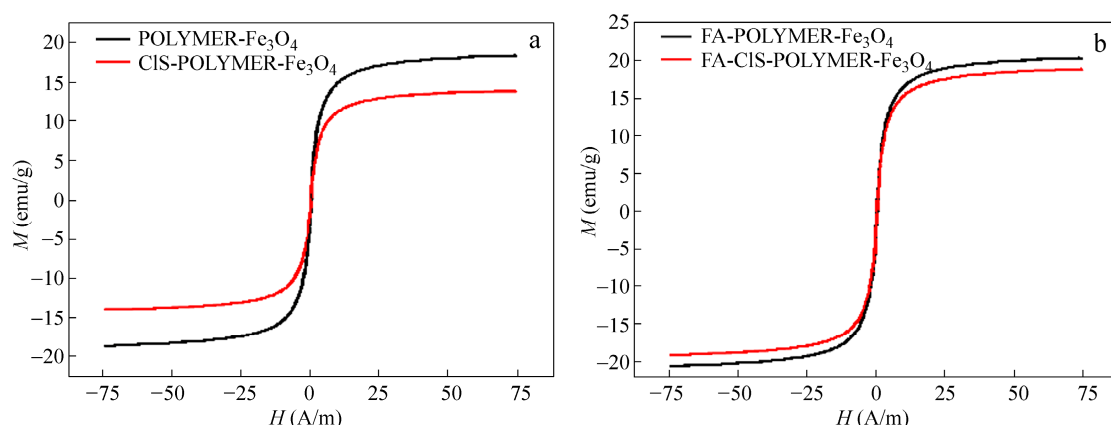


Fig. 3 Room temperature magnetization curves of (a) POLYMER-Fe₃O₄ and CIS-POLYMER-Fe₃O₄, and (b) FA-POLYMER-Fe₃O₄ and FA-CIS-Polymer-Fe₃O₄

Cisplatin Loading and Release

Cisplatin was loaded into the polymer-coated Fe₃O₄ nanoparticles by direct mixing of an aqueous cisplatin solution and a polymer-coated Fe₃O₄ nanoparticle dispersion in phosphate buffer (pH 7.4), followed by dialysis against water. The driving force of the cisplatin loading should be the coordination of the amine groups to the Pt atom in cisplatin. The cisplatin loading capacities of these polymer-coated Fe₃O₄ nanoparticles are shown in Table 2.

The *in vitro* release of the loaded cisplatin was investigated by equilibrium dialysis at 37 °C in PBS, as shown in Fig. 4. The release rate was higher in slightly acidic environment (pH 6.5 and 5.7) than in neutral environment (pH 7.4). The cumulative releases were ~36%, 34% and 22%, respectively, at pH 7.4, 6.5 and 5.7 in 24 h. The release was pH-dependent, which can be attributed to the protonation of the amine groups at lower pHs (Scheme 2). At the more acidic pHs, the more amine groups would be protonated and thereby lost their coordination ability, resulting in the more cisplatin release.

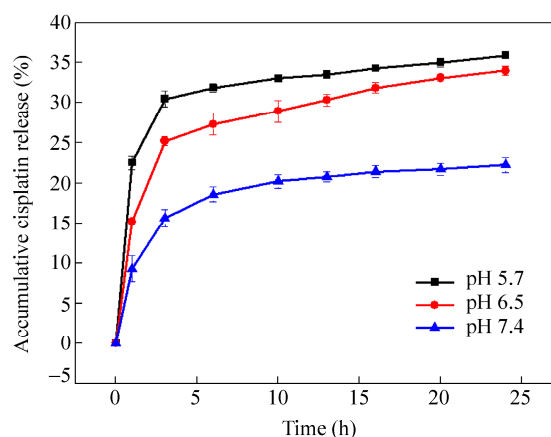


Fig. 4 *In vitro* cisplatin release from CIS-POLYMER-Fe₃O₄ in PBS

Cytotoxicity of Cisplatin-loaded Nanoparticles

All the cisplatin-loaded nanoparticles were cytotoxic to HeLa cells in a concentration-dependent manner, as shown in Fig. 5, indicating that the released drug exhibited anticancer activity. FA-CIS-POLYMER-Fe₃O₄ showed higher cytotoxicity levels than CIS-POLYMER-Fe₃O₄, and similar cytotoxicity with free cisplatin at the same equivalent concentration of cisplatin in the folate-free incubation medium (Fig. 5a). In contrast, FA-CIS-POLYMER-Fe₃O₄ and CIS-POLYMER-Fe₃O₄ exhibited similar cytotoxicity levels, which were lower than that

of free cisplatin at the same equivalent concentration of cisplatin in the incubation medium containing folate (Fig. 5b). These results indicated that the conjugation of folate groups increased the cytotoxicity of the cisplatin-loaded nanoparticles. This increase appeared to be due to the specific targeting of folate receptors overexpressed on HeLa cells.

As a delivery vehicle, low cytotoxicity of a nanoparticle alone is essential. In the absence of drug loading, all the nanoparticles described in this paper showed very low cytotoxicity at concentrations up to 2400 $\mu\text{g/mL}$ (Fig. 6).

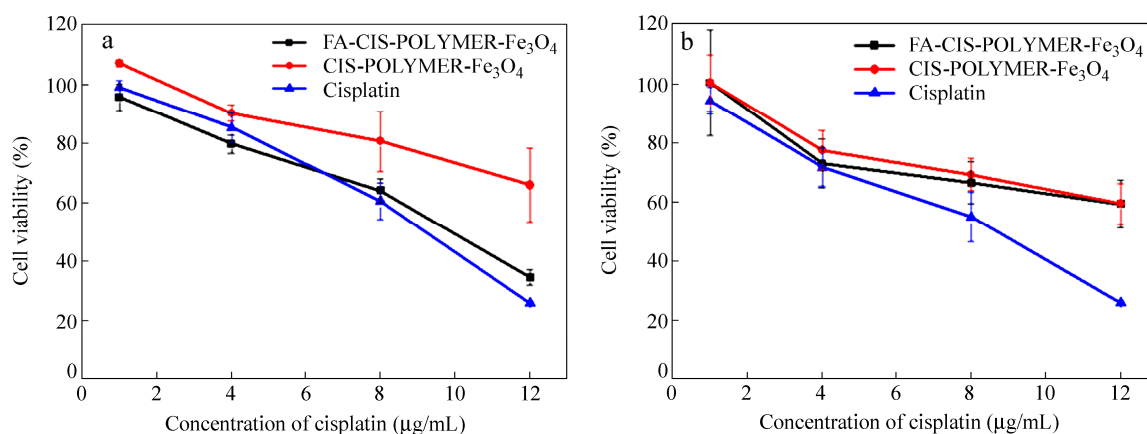


Fig. 5 Viability of HeLa cells incubated with CIS-POLYMER- Fe_3O_4 , FA-CIS-POLYMER- Fe_3O_4 and free cisplatin in (a) RPMI 1640 medium (folic acid free) and (b) RPMI 1640 medium with the addition of folic acid (0.35 mg/mL)

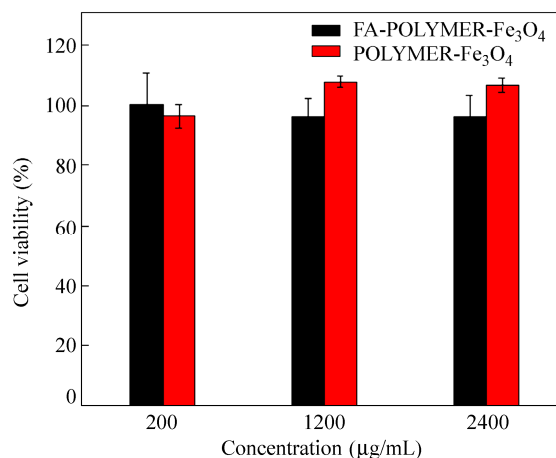


Fig. 6 Viability of HeLa cells incubated with POLYMER- Fe_3O_4 and FA-POLYMER- Fe_3O_4 in RPMI 1640 medium at concentration of 200, 1200 and 2400 $\mu\text{g/mL}$

CONCLUSIONS

In this study we designed and prepared folate-conjugated multilayer core-shell nanoparticles, with superparamagnetic Fe_3O_4 nanoparticles as the core and block copolymer as the shell. The outermost shell was composed of PEG, which imparted biocompatibility to the nanocarriers. Cisplatin was loaded by coordination to the inner shell, which contained amine groups. The loaded cisplatin released faster in more acidic media. Furthermore, folate-conjugated cisplatin-loaded carriers showed ligand-mediated targeting to HeLa cells.

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